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Update on Plant Defense

Plant Defensins: Novel Antimicrobial Peptides as Components of the Host Defense System[1]

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Various mechanisms to fend off microbial invaders have been devised by all living organisms, including microorganisms themselves. The most sophisticated of these mechanisms relies on the synthesis of immunoglobulins directed against specific microbial targets. However, immunoglobulin-based immunity operates only in a relatively minor subset of living species, namely the higher vertebrates. A much more ancient and widespread defense strategy involves the production of small peptides that exert antimicrobial properties. As products of single genes, antimicrobial peptides can be synthesized in a swift and flexible way, and because of their small size they can be produced by the host with a minimal input of energy and biomass. Well-known examples of antimicrobial peptides are the cecropins that accumulate in the hemolymph of many invertebrates in response to injury or infection (reviewed by Boman and Hultmark, 1987) and the magainins that are secreted by glands in the skin of amphibians (reviewed by Bevins and Zasloff, 1990). Cecropins and magainins are small (20–40 residues) basic peptides displaying an amphipathic α-helical structure that can integrate in microbial membranes to form ion channels (Duclohier, 1994).

Another class of antimicrobial peptides is formed by the Cys-rich peptides, which in contrast to cecropins and magainins, have a complex cystine-stabilized three-dimensional folding pattern often involving antiparallel β-sheets. Defensins are one class among the numerous types of Cys-rich antimicrobial peptides, which differ in length, number of cystine, bonds, or folding pattern (reviewed by Boman, 1995). Insect defensins (34–43 residues, three disulfide bridges) are, like cecropins, produced in a pathogen-inducible manner by the insect fat body and secreted in the hemolymph (reviewed by Hoffmann and Hétru, 1992). Mammalian defensins (29–34 amino acids, three disulfide bridges) are produced by various specialized cells in the mammalian body (reviewed by Lehrer et al., 1993; Ganz and Lehrer, 1994). For example, they are very abundant in granules of phagocytic blood cells. These granules fuse with phagocytosis vesicles containing microorganisms, where the defensins are thought to contribute, together with other antimicrobial proteins and active oxygen species, to killing of the engulfed microorganisms. Defensins are also secreted by epithelial cells of the intestines and airways, where they may help maintain the normal microbial flora in a steady state. In addition, the expression of defensins in the airway epithelium has been shown to be up-regulated after exposure to bacterial lipopolysaccharides (Diamond et al., 1993). The importance of defensins in innate immunity of humans is underscored by the observation that certain disorders characterized by recurrent infections are associated with a lack of defensins in blood phagocytes (Ganz et al., 1988). Moreover, transposon mutants of a pathogenic *Salmonella* strain known to infect and grow inside phagocytes simultaneously lost their resistance to defensins (and other antimicrobial peptides) and their virulence (Groisman et al., 1992).

Recently, we characterized a novel class of plant peptides whose structural and functional properties resemble those of insect and mammalian defensins. Hence, we termed this family of peptides "plant defensins" (Terras et al., 1995).

PLANT DEFENSINS SHARE STRUCTURAL PROPERTIES WITH MAMMALIAN AND INSECT DEFENSINS

The first members of the family of plant defensins were isolated from wheat and barley grains by Mendez and co-workers (Collila et al., 1990; Mendez et al., 1990). They were originally called γ-thionins because they showed a similar size (5 kD) and the same number of disulfide bridges (four) as α- and β-thionins. However, subsequent work has established that thionins and γ-thionins are structurally unrelated (Terras et al., 1992; Bruix et al., 1993; Bohlmann, 1994). Proteins homologous to "γ-thionins" have been isolated from seeds of various monocot and dicot species (Bloch and Richardson, 1991; Terras et al., 1992, 1993; Moreno et al., 1994; Osborn et al., 1995) or identified via the sequencing of cDNA clones (Stiekema et

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al., 1988; Ishibashi et al., 1990; Chiang and Hadwiger, 1991; Gu et al., 1992; Karunanandaa et al., 1994).

From a comparison of the amino acid sequences of 14 representative members of the plant defensin family isolated from 13 different species belonging to seven different plant families (Fig. 1), some common features become apparent. Plant defensins are 45 to 54 amino acids long, have a net positive charge, and show clear, although relatively limited, sequence conservation. Residues conserved in all sequences are restricted to the eight Cys's and two Gly's at positions 13 and 34, an aromatic residue at position 11, and a Glu at position 29 (numbering relative to Rs-AFP1).

The three-dimensional structure has been determined by NMR spectroscopy for three members of the plant defensins, namely γ1-P and γ1-H from wheat and barley seeds, respectively (Bruix et al., 1993), and Rs-AFP1 from radish seeds (Fant et al., 1994). Both studies reveal that the structure of plant defensins is dominated by a triple-stranded, antiparallel β-sheet and a single α-helix lying in parallel with the β-sheet. The Cys-X-X-X-Cys segment of the α-helix is connected by two disulfide bridges to the Cys-X-Cys segment in the third β-strand, a structural motif known as the cystine-stabilized α-helix (Kobayashi et al., 1991). A cystine-stabilized α-helix motif also occurs in insect defensins (Bonmatin et al., 1992). As pointed out by Bruix et al. (1992), the global three-dimensional structure of plant defensins closely resembles that of insect defensins, except that insect defensins lack the domain corresponding to the amino-terminal β-strand of plant defensins (Fig. 2). Mammalian defensins, on the other hand, do not feature an α-helix and, hence, lack the cystine-stabilized α-helix motif. However, mammalian defensins do possess a triple-stranded, antiparallel β-sheet (Hill et al., 1991), which is roughly comparable in size and spatial orientation to that occurring in plant defensins (Fig. 2).

Recently, a pathogen-induced peptide with antifungal properties, called drosomycin, was isolated from the fruitfly (*Drosophila melanogaster*) and found to share 38% homologous residues with the plant defensin Rs-AFP1 (Fehlbaum et al., 1994). Considering this remarkable homology, which includes the occurrence of eight Cys's and two Gly's, an aromatic residue, and a Glu residue at positions similar to those of the corresponding conserved residues in plant defensins, we predict that drosomycin shares all structural properties of plant defensins.

Thus, plant defensins belong to a superfamily of similarly folded antimicrobial peptides that has representatives in vertebrates, invertebrates, and plants, suggesting that these defense molecules predate the evolutionary divergence of animals and plants.

SOME PLANT DEFENSINS ARE POTENT INHIBITORS OF MICROBIAL GROWTH

Recent work in our laboratories has established that several members of the plant defensin family inhibit

Species (protein)	Tissue	Sequence
Raphanus sativus (Rs-AFP1)	S	ZKLC-ERPSGTWSGVCGNNNACKNQCINLEK-ARHGSCNYVFPAHKCICYFPC
Raphanus sativus (Rs-AFP2)	S	ZKLC-QRPSGTWSGVCGNNNACKNQCIRLEK-ARHGSCNYVFPAHKCICYFPC
Heuchera sanguinea (Hs-AFP1)	S	DGVKLC-DVPSGTWSGHCGSSSKCSQQCKDREHFAYGGACHYQFPSVKCFCKRQC
Aesculus hippocastanum (Ah-AMP1)	S	LCNERPSQTWSGNCGNTAHCDKQCQDWEK-ASHGACHKRENHWKCFCYFNC
Dahlia merckii (Dm-AMP1)	S	ELC-EKASKTWSGNCGNTGHCDNQCKSWEG-AAHGACHVRNGKHMCFCYFNC
Clitoria ternatea (Ct-AMP1)	S	NLC-ERASLTWTGNCGNTGHCDTQCRNWES-AKHGACHKR-GNWKCFCYFDC
Vigna unguiculata (pSAS10)	S	KTC-ENLVDTYRGPCFTTGSCDDHCKNKEH-LLSGRCRDDV---RCWCTRNC
Pisum sativum (pI230)	Po	NTC-ENLAGSYKGVCFGG--CDRHCRTQEG-AISGRCRDDF---RCWCTKNC
Sorghum bicolor (Siα2)	S	RVC-MGKSAGFKGLCMRDQNCAQVCL-QEG-WGGGNCDGVM--RQCKCIRQC(W)
Triticum aestivum (γ1-P)	S	KIC-RRRSAGFKGPCMSNKNCAQVCQ-QEG-WGGGNCDGPF--RRCKCIRQC
Hordeum vulgare (γ1-H)	S	RIC-RRRSAGFKGPCVSNKNCAQVCM-QEG-WGGGNCDGPL--RRCKCMRRC
Solanum tuberosum (p322)	T	RHC-ESLSHRFKGPCTRDSNCASVCET-ER-FSGGNCHGFR--RRCFCTKPC
Petunia inflata (PPT)	Pi	RTC-ESQSHRFHGTCVRESNCASVCQT-EG-FIGGNCRAFR--RRCFCTRNC
Nicotiana tabacum (FST)	F	REC-KTESNTFPGICITKPPCRKACIS-EK-FTDGHCSLL---RRCLCTKPC
Consensus motif		--C-------Y-G-C-----C---C---E-----G-C--------C-C---C- F W

Figure 1. Amino acid sequences of 14 different plant defensins. S, Seed; Po, pod; T, tuber; Pi, pistil; F, flower. The following sequences were derived from protein sequencing: Rs-AFP1, Rs-AFP2 (Terras et al., 1992, 1995); Hs-AFP1, Ah-AMP1, Dm-AMP1, Ct-AMP1 (Osborn et al., 1995); Siα2 (Bloch and Richardson, 1991; Nitti et al., 1995); γ1-P (Colilla et al., 1990); and γ1-H (Mendez et al., 1990). The following sequences were deduced from cDNA clones with omission of the putative signal peptides: pSAS (Ishibashi et al., 1990); pI230 (Chiang and Hadwiger, 1991); p322 (Stiekema et al., 1988); PPT (Karunanandaa et al., 1994); and FST (Gu et al., 1992). The carboxyl-terminal Trp of Siα2 is uncertain, according to Nitti et al. (1995). The antimicrobial peptide Pth-St1 isolated from potato tubers differs from the p322-derived sequence by only a single substitution in the second residue (His to Asn) within the first 20 residues (Moreno et al., 1994). The sequence of FST shown is restricted to the Cys-rich domain and does not include the acidic carboxyl-terminal propeptide domain.

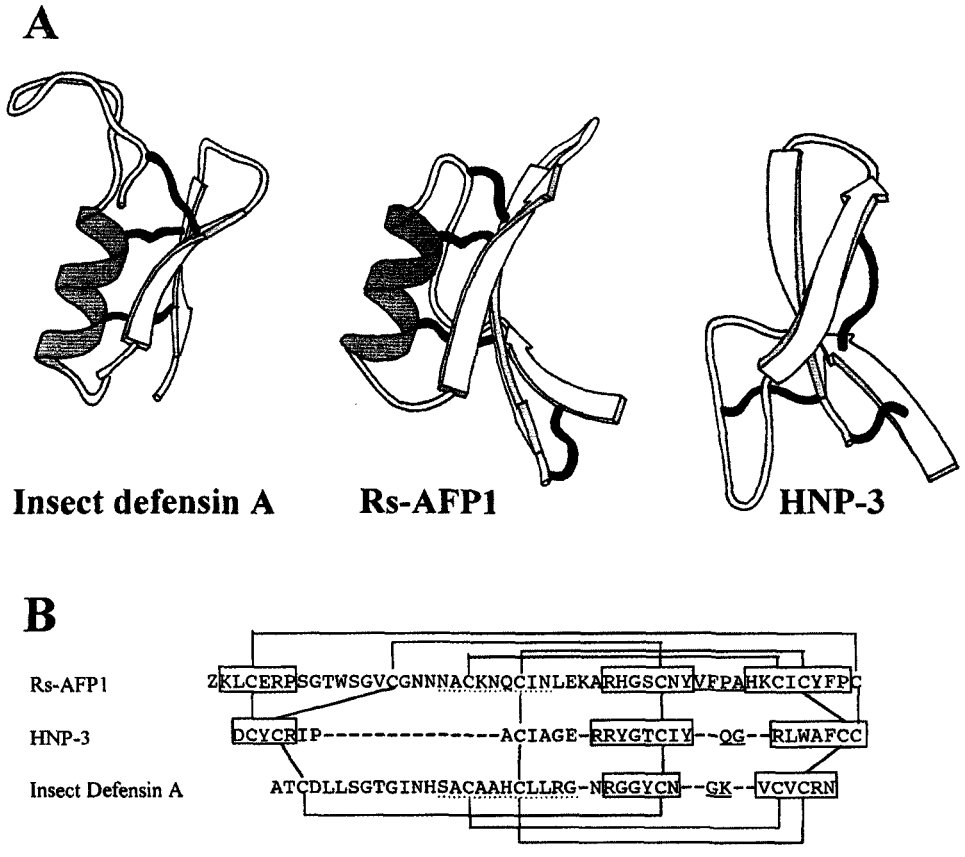

Figure 2. A, Schematic representation of the three-dimensional structure of a plant defensin (Rs-AFP1; Fant et al., 1994), an insect defensin (Insect defensin A; Bonmatin et al., 1992), and a mammalian defensin (HNP-3; Hill et al., 1991). β-strands are represented by yellow arrows, α-helices by red helical ribbons, and disulfide bridges by black tubes. B, Comparison of the amino acid sequences, disulfide bridges, and secondary structure elements of plant defensins (Rs-AFP1), insect defensins (Insect defensin A), and mammalian defensins (HNP-3). Gaps introduced for optimal alignment are indicated by dashes. Boxed sequences represent β-strands and underlined sequences represent β-turns; sequences underlined by a dashed line correspond to α-helices. Disulfide bridges are indicated by the lines connecting Cys pairs.

growth of a broad range of fungi at micromolar concentrations. At least two groups of antifungal plant defensins can be distinguished according to the morphogenic effects caused on treated fungal hyphae. The "morphogenic" plant defensins cause reduced hyphal elongation with a concomitant increase in hyphal branching. The plant defensins from the Brassicaceae (including Rs-AFP1 and Rs-AFP2 from radish seeds) and Saxifragaceae (including Hs-AFP1 from *Heuchera sanguinea* seeds) belong to this group (Terras et al., 1992, 1993; Osborn et al., 1995). On the other hand, the plant defensins from the Asteraceae (including Dm-AMP1 from dahlia seeds), the Fabaceae (including Ct-AMP1 from *Clitoria ternatea* seeds), and the Hippocastanaceae (including Ah-AMP1 from horse chestnut seeds) slow down hyphal extension but do not induce marked morphological distortions. These peptides are tentatively termed "nonmorphogenic" plant defensins. Morphogenic and nonmorphogenic plant defensins also differ in their antifungal spectrum. For instance, in a medium consisting of potato dextrose broth including 1 mM $CaCl_2$ and 50 mM KCl, the nonmorphogenic plant defensins are markedly more active than the morphogenic plant defensins against *Leptosphaeria maculans*, whereas the opposite is true for *Penicillium digitatum* (Osborn et al., 1995). Bacteria are mostly unaffected by plant defensins, with some exceptions such as *Bacillus subtilis*, which is inhibited by Ct-AMP1 (Osborn et al., 1995), and *Pseudomonas solanacearum* and *Clavibacter michigansis*, which are susceptible to Pth-St1, a plant defensin isolated from potato tubers (Moreno et al., 1994). None of the plant defensins has to date been found to affect the viability of human or plant cells (Terras et al., 1992; W.F. Broekaert and B.P.A. Cammue, unpublished results).

The antifungal activity of plant defensins, whether morphogenic or not, is reduced by increasing the ionic strength of the fungal growth assay medium. However, the antagonistic effect of ions is strongly dependent on the fungus and thus on the conformation of the putative target site (Terras et al., 1992; Osborn et al., 1995). Ionic strength antagonism was found to be due to cations, with divalent cations being at least 1 order of magnitude more potent than monovalent cations (Terras et al., 1992, 1993). In general, the antifungal activity of plant defensins is slightly more reduced by Ca^{2+} than by Mg^{2+} (Osborn et al., 1995).

Reduction of the antimicrobial activity in the presence of divalent cations, especially Ca^{2+}, has also been observed for insect defensins (Cociancich et al., 1993) and mammalian defensins (Lehrer et al., 1988).

A third subclass of plant defensins is formed by their homologs found in the Poaceae, including γ1-P, γ1-H, and Siα1–3 from wheat, barley, and sorghum seeds, respectively. These plant defensins belong to the "α-amylase inhibitor" type because they have been reported to inhibit α-amylases from insects and humans (Bloch and Richardson 1991; Osborn et al., 1995). At least γ1-P, Siα1, Siα2, and Siα3 were shown to be not or very weakly active against fungi (Osborn et al., 1995). Conversely, antifungal plant defensins such as Pth-St1 (Moreno et al., 1994), Ah-AMP1, Ct-AMP1, Dm-AMP1, Hs-AFP1, and Rs-AFP2 (Terras et al., 1992; Osborn et al., 1995) do not exert α-amylase inhibitor activity. The α-amylase inhibitor-type plant defensins may possibly have evolved away from a role in protection against microorganisms toward protection against herbivores. Recent experiments with transgenic plants have established that the expression of α-amylase inhibitors in seeds confers protection against some seed-feeding insects (Shade et al., 1994).

PLANT DEFENSINS AND THEIR ROLE IN THE PROTECTION OF SEEDS

We have studied the release of antimicrobial peptides from germinating radish seeds using a simple bioassay in which seeds are allowed to germinate on a medium supporting the growth of a fungal colony (Terras et al., 1995). Radish seeds germinating on such a medium caused a growth inhibition halo in the fungal colony, which was not apparent when proteases were included in the medium. The inhibition halo was also formed around drops containing as little as 1 μg of either of the purified radish plant defensins, Rs-AFP1 or Rs-AFP2. Seeds that were kept dormant by external application of ABA did not produce the inhibition halo unless their seed coats were mechanically perforated. Analysis of the imbibition solution of seeds with a mechanically incised seed coat revealed that Rs-AFPs accounted for 30% of released proteins, although Rs-AFPs are minor proteins in the seed (0.5% of total seed proteins). The amount of Rs-AFPs released from a single seed was estimated to be at least 1 μg, which is the amount of peptide required to mimic the inhibition halo formed around a germinating seed. All of these experiments indicate that Rs-AFPs are released from radish seeds when the seed coat is perforated (either by the radicle of the germinating embryo under natural conditions or artificially with the aid of a scalpel), and moreover, that the released amounts are sufficient to create a zone around the seeds in which fungal growth is suppressed. Hence, these findings strongly suggest that Rs-AFPs play a role in the protection of seedling tissues during the early stage of emergence and thus may contribute to the enhancement of seedling survival rates. The simple fact that chemical fungicides are commonly used for the coating of crop seeds to increase seedling stand illustrates that soil-borne or seed-borne fungi form a considerable threat to germinating seeds.

The preferential release of Rs-AFPs from imbibing seeds is consistent with their localization pattern as determined by immunocytochemistry (Terras et al., 1995). Rs-AFPs were demonstrated by EM to be located in the cell walls, more precisely in the middle lamellae. The extracellular location of Rs-AFPs is also underscored by the fact that the Rs-AFP1 cDNA encodes a preprotein. Although Rs-AFPs were found throughout the endosperm, cotyledon, and hypocotyl, they were much more abundant in the outer cell wall lining the periphery of these organs. This implies not only cell-specific regulation of expression but also a polarity in the deposition at the subcellular level. The surface walls of endosperm, cotyledon, and hypocotyl are the first to be hydrated when the seed starts imbibing water, which may explain the preferential release of Rs-AFPs from this location.

PLANT DEFENSINS AND THEIR ROLE IN THE PROTECTION OF VEGETATIVE TISSUES

Although most plant defensins isolated to date are seed derived, evidence is now accumulating that plant defensins are also expressed in vegetative tissues. In radish plants, 5-kD peptides cross-reacting with antibodies raised against Rs-AFP1 were found at low concentrations in the leaves, but they accumulated upon infection with *Alternaria brassicola* both in infected and uninfected leaves (Terras et al., 1995). Likewise, transcripts hybridizing to a Rs-AFP1 cDNA clone were detected in healthy leaves, but their steady-state levels strongly increased systemically upon challenge inoculation of leaves with either *A. brassicola* or *Botrytis cinerea* or upon treatment of the leaves with mercuric chloride (Terras et al., 1995). Two Rs-AFP homologs were purified from *A. brassicola*-infected radish leaves and shown to share about 90% amino acid sequence homology to the seed Rs-AFPs and to cause similar morphological distortions on fungal hyphae (Terras et al., 1995).

A study of pathogen-induced transcripts in pea pods led to the identification of two cDNA clones, called pI39 and pI230, whose corresponding transcripts accumulated upon inoculation of the pods with either a compatible (*Fusarium solani* f.sp. *pisi*) or incompatible (*Fusarium solani* f.sp. *phaseoli*) fungal pathogen (Chiang and Hadwiger, 1991). Both cDNA clones encoded preproteins with a signal peptide domain and a plant defensin domain. It is interesting that one of these cDNA clones, pI39, also corresponds to an epidermis-specific transcript that was isolated by differential screening of pea cDNA libraries prepared from leaf epidermis and the remainder of the leaf (L. Press, G. Stewart, and J. Manners, personal communication). Thus, the corresponding pea gene appears to be constitutively expressed in the leaf epidermis and transcriptionally upregulated upon fungal attack in pods and possibly also in other vegetative tissues. It remains to be demonstrated, however, whether or not pathogen-responsive expression alters the spatial expression pattern of this plant defensin gene.

A tobacco cDNA clone referred to as FST was characterized by Gu et al. (1992) and encodes a protein with a signal peptide domain, a plant defensin domain, and a 33-residue

carboxyl-terminal domain featuring clusters of hydrophobic and acidic residues. The carboxyl-terminal domain of FST is cleaved off during processing (A. Cheung, personal communication) and might function as a determinant for transport to the vacuole (Vitale and Chrispeels, 1992) or, alternatively, interact with the cationic plant defensin domain to prevent association with components of the intracellular protein-trafficking system. In healthy tobacco plants, transcripts hybridizing to the FST cDNA were found only in unopened flower buds. In situ hybridization revealed that the transcripts were most abundant in the epidermis of the adaxial surface of the petals and in the peripheral cell layers of the style, the ovary, the stamen filaments, and anthers. In healthy leaves and sepals, transcripts were undetectable, but they accumulated in these organs upon fungal infection (Gu et al., 1992; A. Cheung, personal communication).

The expression of the gene encoding Pth-St1, the antimicrobial plant defensin from potato tubers, was shown to be most abundant in the epidermal cell layer and leaf primordia of the tuber (Moreno et al., 1994). The same or a related gene is also expressed in flowers, stems, and leaves.

The common picture arising from these studies is that plant defensins are expressed either in a constitutive and organ-specific manner, in which case they are most abundant in peripheral cell layers of that organ, or in a pathogen-modulated manner, possibly associated with altered tissue specificity. The constitutive expression of plant defensins in peripheral cells of seeds (in the case of radish), flowers organs (in the case of tobacco), leaves (in the case of pea), or tubers (in the case of potato) is consistent with a role in first-line defense of vulnerable tissues.

To investigate more directly the ability of plant defensins to control fungal pathogens in planta, we have transformed tobacco plants with a chimeric gene construct, consisting of a constitutive enhanced cauliflower mosaic virus 35S promoter fused to the coding region of the Rs-AFP2 preprotein. T_2-generation tobacco plants expressing Rs-AFP2 at a level of 0.2% of total leaf proteins showed a 7-fold reduction in lesion size upon infection with the foliar fungal pathogen *Alternaria longipes* relative to untransformed plants (Terras et al., 1995).

CONCLUSION

Plant defensins represent a novel class of antimicrobial peptides showing structural and functional homology to their counterparts in animals, the insect and mammalian defensins, whose involvement in host defense is well established. Several lines of evidence support the notion that plant defensins are important components of the defense system in plants as well, including their location at the periphery of different organs, their induction under pathogenic stress conditions, and the demonstration that transgenic plants constitutively expressing a plant defensin show enhanced disease resistance.

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